1.0 Purpose:

This study will evaluate a variety of procedures for extracting *Legionella* from an open-cell foam Environmental Indicator Device.

2.0 Scope:

To identify an effective method for the extraction and detection of *Legionella* spp. from an open-cell foam Environmental Indicator device (Open-cell Sponge). Separate Open-cell Sponge devices will be indirectly inoculated with fresh *Legionella* cultures at high, medium, and low concentrations and evaluated using a combination of various surfactants, extraction procedures, and selective media for the detection and identification of *Legionella* spp.

3.0 Test Material:

Test Product:
Open-cell foam Environmental Indicator device and 250 mL glass vial; minimum of 9 test samples.

4.0 Test Microorganisms:

4.1 Mixed organism suspension

a. *Legionella pneumophila* ATCC 33152
b. *Legionella dumoffii* QL14012-1A
c. *Legionella micdadei* QL145022-1A

5.0 Equipment/Supplies:

5.1 Vortex mixer
5.2 Sterile Tap Water
5.3 Sterile blade or scalpel
5.4 Sterile forceps
5.5 Sterile gloves
5.6 Centrifuge
5.7 Robot Coup Blixer 4v Viable Speed Mixer
5.8 Sterile Blender Jar
5.9 Buffered Charcoal Yeast Extract (BCYE) agar (MP359)
5.10 Buffered Charcoal Yeast Extract agar with polymyxin B, cycloheximide, and vancomycin (PCV) agar (MP362)
5.11 Buffered Charcoal Yeast Extract agar with polymyxin B, cycloheximide, vancomycin and glycine (GPCV) agar (MP360)
5.12 Buffered Charcoal Yeast Extract agar with polymyxin B, cycloheximide, and vancomycin without L-cysteine (PCV(-)) agar (MP361)
5.13 Incubator, thermostatically-controlled, maintained at 35 ± 2°C
5.14 Serological pipets, sterile
5.15 Dissecting microscope with oblique lighting

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5.16 Latex Agglutination test kit (Microgen Bioproducts latex agglutination kit M45, or equivalent)
5.17 Bruker MS Biotyper
5.18 10% Polysorbate 80 (Tween 80)
5.19 1% Sodium Lauryl Sulfate
Note: Additional Surfactants may be evaluated upon further development of the method

6.0 Test Microorganism Preparation and Inoculation

6.1 Inoculation of the Open-cell Foam Environmental Indicator Device
- 6.1.1 In replicates of three prepare 1 L spiked water samples inoculated at 1-10, 10-100 and 100-1000 CFU/mL, representing low, mid, and high contamination levels respectively.
- 6.1.2 Add an open-cell sponge device to each beaker. To simulate real world applications of the product, submerge and mix with a sterile forceps for 3-5 minutes.
- 6.1.3 After mixing, place the open-cell sponge into the supplied 250 mL glass vial and fill with 200 – 225 mL of the inoculated water used in the previous step and tighten the cap on the glass jar. Allow the sample to sit for 24 hours at ambient temperature (20 - 24°C).

7.0 Preparation of Specimens for Bacteriological Examination

7.1 Sample Extraction
- 7.1.1 After 24 hours, remove vial cap and pour sample water into a sterile blender jar.
- 7.1.2 Wearing sterile gloves and using a sterile scalpel cut the plastic zip tie and remove it along with the metal ring from the open-cell sponge using sterile forceps. Place all the foam strands of the open-cell sponge into the sterile blender jar containing the sample water. To assist in the removal of bacteria from the open-cell sponge foam strands, add 1 mL of 10% surfactant (Tween 80) solution.
- 7.1.3 If Tween 80 is not conducive to removing Legionella from the sample devices, steps 6.1.2 – 7.1.2 will be repeated using a different surfactant (i.e. substituting 1% Sodium Lauryl Sulfate for Tween 80).
- 7.1.4 Cap the blender jar and blend contents at the highest setting for 2 minutes. Set aside sample containers at ambient temperature to allow large particulates to settle for at least 10 minutes.
- 7.1.5 Carefully transfer the supernatant to a 250 mL falcon tube, leaving enough supernatant to avoid transferring large particulates of the homogenized sponge.
- 7.1.6 Centrifuge samples at 5500 x g for 30 minutes and discard all but the last 5 mL of the supernatant into a biohazard container.
- 7.1.7 Additional extraction procedures will be evaluated if the homogenization step is not effective

7.2 Sample Plating
- 7.2.1 Vortex the remaining supernatant and spread plate 0.1 mL onto 1 BCYE, 2 PCV, 2 GPCV and 1 PCV (-) media. The remaining sample will be capped and stored at 4°C until after data collection and analysis.
- 7.2.2 Incubate plates at 35 ± 1°C in a humidified incubator for 72 to 96 hours. Plates may be incubated for up to 7 days before being deemed absent of *Legionella*.

7.3 Examination of Selective Agars

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7.3.1 Macroscopic examination of the media will be performed with a dissecting microscope and oblique lighting to detect colonies resembling those of *Legionella.*

7.3.2 Positive confirmations will be confirmed via latex agglutination and identification using the Bruker MS Biotyper.

7.3.3 If the originally selected surfactants evaluated are not effective, additional surfactants will be evaluated for the product method.

8.0 Study Controls:

Study controls will include a positive water sample (spiked) without the open-cell sponge, and two negative water samples, one with and one without the open-cell sponge.

9.0 Final Report:

Interim results of the progress of the method evaluations will be provided to the study sponsor

A Final Report will be prepared upon completion of the study, including a tabularized summary of data and a description of results of the study.

10.0 Documentation and Record-Keeping:

All documentation and records will be compiled, analyzed, and retained by Q Laboratories, Inc. at its facility in Cincinnati, Ohio. All raw data for this study, as well as the Final Report, will be sent to the study sponsor and retained in safe storage by the Testing Facility for a period of at least seven (7) years (20 –ADMN-ISO-008D, Control of Records).

11.0 Quality Compliance:

Q Laboratories, Inc. has developed and implemented a quality management system that enhances our ability to provide testing services that consistently meet client expectations and regulatory requirements. Q Laboratories, Inc. quality documentation requirements are defined by ISO 17025, FDA Quality System Regulations (QSR), FDA Current Good Manufacturing Practices (cGMPs), FDA Good Laboratory Practices (GLP) and EPA Good Laboratory Practices standards (GLPs).

Q Laboratories, Inc. applies the following standards as applicable:

- ISO 17025:2005 General Requirements for the Competence of Testing and Calibration Laboratories
- FDA 21 CFR Part 820 Quality System Regulation
- FDA 21 CFR Part 58 Good Laboratory Practice For Non Clinical Laboratory Studies
- FDA 21 CFR Part 211 Current Good Manufacturing Practice for Finished Pharmaceuticals
- FDA 21 CFR Part 210 Current Good Manufacturing Practice in Manufacturing Processing, Packing or Holding of Drugs; General
- EPA 40 CFR Part 160 FIFRA Good Laboratory Practice Standards

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Periodic phase audits of the study will be conducted by the Quality Assurance Unit to ensure testing compliance as per section 58.35 (b) (3) of 21 CFR. A review of the final report by the QAU will be conducted in accordance with 21 CFR, part 58.185, subpart J.

12.0 Protocol Modifications:

During the testing phase, changes to the protocol may be required. The study sponsor will be notified immediately of any modifications to the protocol. Approval of the modifications are required before any additional analysis is conducted. The modifications will be added to the protocol as an amendment and approved by both the study director and study sponsor.

13.0 Product Disposition:

It is the responsibility of the Study Sponsor to retain a sample of the test substance(s) for future audit or evaluation. All unused test material will be disposed within 90 days following the study completion unless otherwise indicated by the Study Sponsor prior to initiation of the study.

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